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Synthesis and preliminary characterization of a new species (amphidiploid) in *Cucumis*

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Summary

A successful interspecific hybridization between cucumber (*Cucumis sativus* L., 2n = 14) and a wild *Cucumis* species, *C. hystrix* Chakr. (2n = 24) was made via embryo rescue. Hybrid plants (2n = 19; 7 from cucumber and 12 from *C. hystrix*) were sterile, but morphologically uniform. Self-pollination and backcrossing of F_1 hybrid plants to either parent confirmed presence of both male- and female-sterility that were likely caused by lack of homology and improper pairing during meiosis. While the multiple-branching habit, densely brown hairs (on corolla and pistil), orange-yellow corolla, and ovate fruit of F_1 hybrid plants were similar to that of the *C. hystrix* parent, the appearance of the first pistillate flower was more similar to that of *C. sativus* parent. The diameter and internode length of the stem, and the shape and size of leaves and flowers were intermediate when compared to the parents. The chromosome number in the hybrid was doubled through somaclonal variation during embryo culture and regeneration process to restore the fertility. Pollen grains were released and fruits with viable seeds matured on fertile, synthetic amphidiploid plants. The results from flow cytometry indicated that, on average, 7.3% of the morphologically unique regenerants had the 4C DNA content of 2.35 pg relative to the 2C DNA content of the original F_1 hybrid at 1.17 pg and, therefore, were likely chromosome-doubled F_1 hybrids (2n = 38). Nutritional analysis indicated that the synthetic species had higher protein (0.78%) and mineral (0.35%) content compared to the normal pickling cucumber (0.62% and 0.27%, respectively), and could be considered a new *Cucumis* crop having a special place in the future agriculture. Preliminarily evaluation indicates that *C. hystrix* possesses a high level of root-knot nematode resistance, and that this resistance is partially expressed in the interspecific F_1 and chromosome-doubled F_1. This and the fact that the fruit morphology of the fertile amphidiploid differs during the growing season (e.g., short and long fruit) suggest that it could be useful in broadening the germplasm base of cucumber.

Introduction

When applicable, interspecific hybridization is the most efficient way to transfer useful characters from wild relatives to the cultivated species (Stalker, 1980). Allopolyploids, such as allotetraploids or amphidiploids, are developed by interspecific hybridization and chromosome doubling of the initial F_1 hybrid. Successful construction of an allopolyploid means the creation of a new combination of genomes, and consequently, perhaps a new species if the cross is extremely wide and the resultant progeny are unique.

Often, considerable effort is required to obtain an interspecific hybrid, especially between cultivated and wild species. Chromosomal, genetic, cytoplasmic, or mechanical isolation barriers can present severe handicaps for successful hybridization and utilization. In the Cucurbitaceae, an amphidiploid was reported from

a cross between *C. maxima* × *C. moschata* (Pearson et al., 1951), and a mating between *C. anguria* × *C. dipsaceus* (Yadava et al., 1986). There has been no successful interspecific hybridization between cucumber and melon which are the two commercially most important *Cucumis* species.

The importance of wild relatives for cucumber improvement has long been recognized (Fassuliotis, 1967; Norton & Granberry, 1980). They possess resistance to pathogens such as powdery mildew (*Sphaerotheca fuliginea*), downy mildew (*Pseudoperonospora cubensis*), anthracnose (*Colletotrichum orbiculare*), fusarium wilt (*Fusarium oxysporum*), and root-knot nematode (*Meloidogyne spp.*). Due to cross-incompatibility, various approaches (classical and biotechnological) have been used to introduce genes from wild relatives (Whitaker, 1930; Batra, 1953; Smith & Venkat Ram, 1954; Deakin et al., 1971; Fassuliotis & Nelson, 1988). These attempts have been largely unsuccessful. The interspecific hybridization made between *Cucumis sativus* and *C. hystrix* (Chen et al., 1997) is the first successful interspecific hybridization in *Cucumis.* Although this hybrid offered the possibility of introduction of desirable characters from *C. hystrix* to cultivated *C. sativus,* reciprocal F_1 hybrids were sterile. Sterility was likely due to the odd chromosome number in hybrid plants (2n = 19 chromosomes; n = 7 from *C. sativus* and n = 12 from *C. hystrix*). In most cases, the sterility is associated with meiotic abnormalties. Preliminary experiments indicated that the fertility could be overcome by chromosome doubling. Fertility restoration in the synthetic amphidiploid (2n = 38) might result in homologous pairing at meiosis, thus producing viable pollen and egg cells. The present paper reconfirms the production of the synthetic *Cucumis* species through interspecific hybridization and chromosome doubling, and provides for its characterization based on morphological, nutritional, and resistance attributes.

Materials and methods

Plant material and interspecific hybridization

An accession of *C. hystrix* and two cultivars of *C. sativus* were used. Seeds of *C. hystrix* were collected in South China (Chen et al., 1994). North Chinese cucumber cultivar 'Beijing Jietou' was received from Dr C.Z. Qi, of the Vegetable Research Institute, Chinese Agricultural Academy of Sciences, Beijing, and the South Chinese cucumber cultivar 'Er Zhaozi' was received from Mr Z.B. Gong, of the Chengdu Seed Company #2, Chengdu city, Sichuan. Seedlings of *C. hystrix* were transplanted to the field between June and October, *C. sativus* was grown in the greenhouse between July and November. The interspecific crosses were made between September and October 1997, using the procedure described by Chen et al. (1997).

Embryo culture and regeneration procedure

The fruits were harvested 30 days after pollination and surface-sterilized with 70% ethanol prior to the extraction of seeds. Excised embryos were placed immediately on MS (Murashige & Skoog, 1962) hormone-free medium containing 30 g/L sucrose and 8 to 12 g/L agar at pH 5.6 and cultured at 24 °C under fluorescent lighting at 16h light ($\sim 100 \mu mol \cdot m^{-2} \cdot S^{-1}$): 8h dark photoperiod for about 60 days until plantlets formed. After 90 days, the edges of the leaf tissue in the plantlets were cut and sliced into 0.5 cm pieces as explants. The explants were placed abaxial side-up on a MS medium containing Agar 8 g/L, Sucrose 30 g/L, 1.0 mg/L 2.4-D, 0.5 mg/L BA, 0.4 mg/L ABA, and 10 mg/L $AgNO_3$. After four weeks incubation under lights, they were transferred to the MS hormone-free medium. In 3 to 4 weeks (after callus and shoots appeared), the shoots were excised and transferred to fresh MS medium with 1.0 mg/L IBA to form roots. Once small roots had developed, the plantlets were gently removed from agar, rinsed with water, and placed into moist vermiculite. The plants were acclimatized under a plastic dome (40 cm × 60 cm) for about one week and grown in the greenhouse.

Measurement of morphological traits

Greenhouse-grown hybrid and parental plants were evaluated for morphological traits at maturity. The appearance of the first flower as well as length of the pedicel, calyx tube, corolla and ovaries was recorded on each of five plants. Stem diameter and internode length were measured at the 1^{st}, 5^{th}, 10^{th}, 15^{th}, and 20^{th} node of each of five plants. Petiole length of five plants was recorded at node 20. The size of leaves subtending petioles was estimated by comparison of weight measurements of samples to a known standard having similar mass. Weight of each sample was compared to a standard whose, leaf area ratio could be determined and leaf area estimated. Means and standard deviations were calculated for each trait measured.

Table 1. Morphological characteristics differentiating *Cucumis sativus* ('Beijing Jietou'), *C. hystrix* and their F_1 and chromosome-doubled F_1 hybrid progeny

Traits		Cultigens			
		C. hystrix	*C. sativus*	F_1	Doubled F_1
Chromosome number		24	14	19	38
Stem	– diameter[a] (cm)	0.3± 0.1	0.6± 0.2	0.4± 0.2	0.5± 0.1
	– internode length[a] (cm)	4.8± 2.7	10.6± 2.4	4.8± 1.2	5.7± 1.4
	– no. of lateral branches[b]	12.0± 2.0	2.6± 1.4	5.4± 1.2	5.8± 1.4
Leaf[a]	– length of petiole (cm)	7.0± 1.0	12.6± 1.4	9.8± 5.2	8.8± 3.2
	– leaf size (cm2)	102.1±13.9	321.5±34.5	191.2±27.8	276.4±36.2
Staminate					
flower	– appearance of first flower[c]	15.0±4.0	3.8±1.3	6.8±1.2	5.6±1.6
	– length of pedicel[d] (cm)	0.5±0.2	2.4±0.8	0.5±0.1	0.5±0.2
	– length of calyx tube[d] (cm)	0.5±0.1	0.8±0.1	0.6±0.2	0.7±0.2
	– corolla length[d] (cm)	0.9±0.1	2.5±0.2	1.2±0.1	1.2±0.2
Pistillate					
flower	– appearance of first flower[c]	–	4.2±1.8	5.2±2.0	–
	– ovary length×diameter[d]	1.0×0.4	3.8×0.7	1.5×0.4	1.6×0.7
	– corolla length[d]	1.0±0.1	2.6±0.2	1.3±0.4	1.5±0.2
Fruit	– length×diameter (cm)	5.5×2.2	25×4.5	9.2×3.2	8.1×4.0
	– weight (g)	–	–	54.4	90.6
Seed	– length×width(cm)	0.3×0.2	1.0×0.4	–	0.67×0.3
	– thickness (cm)	0.07±0.01	0.16±0.01	–	0.15±0.01

[a] Commulative average measurement taken at the 1st, 5th, 10th, 15th, and 20th node of each of five plants.
[b] Average number to the 20^{th} node of five plants.
[c] Average of five plants for days to first flower.
[d] Average of five flowers from each of five plants.

Ploidy determination by flow cytometry

Newly expanded leaf tissues of the plantlets from tissue culture were cut into small pieces in $MgSO_4$ buffer. After washing with 1 × PBS buffer, the suspension of nuclei was filtered through one layer of mira cloth with pore size 15 μm. The nuclei were adjusted to the concentration of 2×10^5 nuclei/ml prior to staining with propidium iodide (PI) in a solution containing RNase. Nuclei were analysed with the flow cytometer Epics 751 (Coulter Corporation). Excitation of PI was provided by the 488 nm line (400 mW) of an argon laser (model I-90, Coherent) and the red fluorescence emitted by PI was collected through a 635 nm band pass filter. Chicken red blood cells (CRBC) with DNA content of 1.88 pg were used as an internal standard. Each sample was analysed both with and without the standard to detect ploidy level. The data were presented as histograms, and the nuclear DNA content was calculated as: 1.88 × (position of plant nuclear peak/position of CRBC nuclear peak).

Nutritional analysis

Nutritional analysis was carried out according to AOAC (Association of Official Analytical Chemists) procedures. The 'Kjeldahl' method was used for nitrogen determination, using 6.25 as the conversion factor; 'drying' was used for moisture, and ashing was used to make an incinerated product (using a muffle furnace), and carbohydrate was calculated by differences.

Screening for resistance to southern root-knot nematode

Meloidogyne incognita race 3 was collected from a home vegetable garden. It was cultured on greenhouse-grown tomato, *Lycopersicon esculentum* Mill. cv. Rutgers. Nematode inoculum was obtained by collecting eggs with 0.5% NaOCl as described by Hussey & Barker (1973).

Seeds were germinated in vermiculite in a greenhouse, and seedlings at the two-leaf-stage were transplanted into 4-inch pots filled with pure sand. Plantlets from *in vitro* culture were also transplanted into the

same medium at the same time. Plants were fertilized weekly with a commercial nutrient formulation (N: P: K = 20: 20: 20), and kept in a greenhouse at about 28 °C. Four days after planting, two holes 20 to 30 mm in depth and 6 mm in diam. were made with a bamboo stick around the plant roots. One ml of inoculum containing 2,500 nematode eggs was pipetted into each hole (5,000 eggs per plant). There were five replications of each cultigen to determine the ability of the nematode reproduce. Plants were placed on a growing table in a completely randomized design. Seven weeks after inoculation, the root systems were carefully washed free of sand, and evaluated for number of galls under a stereoscopic microscope at 10 × magnifications. The number of galls for each root system was counted, and a gall index was calculated using a 0–5 scale; where 0 = no galls, 1 = 1 or 2, 2 = 3–10, 3 = 11–30, 4 = 31–100, and 5 > 100 galls.

Results

Production and morphology of the hybrids

All pollinations resulted in mature fruit having seeds containing heart-shaped embryos. Fifty-nine of the 159 rescued embryos (about 37.3%) developed into whole plants. In the 854 plantlets obtained through organogenesis, 62 plants (7.3%) demonstrated unique characters in morphology, and were later confirmed as chromosome-doubled F_1 hybrids.

The F_1 hybrid plants grew vigorously and were morphologically uniform (Table 1). The multiple branching habit, dense brown hairs (especially on corolla and pistil), orange-yellow corolla and ovate fruit of hybrid plants were similar to that of the *C. hystrix* paternal parent. The appearance of the first pistillate flower in hybrid plants was more similar to that of the *C. sativus* maternal parent than to *C. hystrix*. However, the appearance of staminate flowers, and the diameter and the internode length of the main stem, and shape and size of the leaves (Figure 1a), flowers (Figure 1b) and fruits (Figure 1c) were intermediate.

The chromosome-doubled F_1 plants were distinct from the parents and other progeny in morphology in such traits as a curve on leaf margins, and shorter and stronger internodes. The fruits at two ploidy levels vary in morphology (Figure 1d). While diploid fruit (2n = 19, seedless) was longer and spindle-like in shape, the tetraploid fruit was shorter and column-shaped. The seeds from the synthetic species were intermediate in size and shape compared to the diploid progenitors (Figure 1e).

Flow cytometry

The results from flow cytometry confirmed the fact of chromosome doubling (Figure 2). The peak position of G0/G1 nuclei was used to calculate 2C nuclear DNA content. Based on the DNA content of internal standard CRBC 1.88 pg, the DNA content of the original F_1 hybrid was 1.17 pg, and the chromosome-doubled F_1 hybrid was 2.35 pg.

Considerable differences in the rate of chromosome doubling were observed between the two genotypes used. When the hybrid derived from 'Beijing Jietou' was used as seed parent, 40 chromosome-doubled plants were obtained from 761 regenerants (5.3%). In contrast, when 'Er Zhaozi' was used as the seed parent for interspecific hybridization, 22 chromosome-doubled plants were obtained from 93 regenerants (23.7%).

Nutrition analysis

The results from nutrition analysis (Table 2) indicated that the percentage of ash (0.35%) and protein 0.78%) in the interspecific hybrid were higher than in the control. However, the control had higher percentage of carbohydrate (3.51%) than the sample (2.47%). The lower PH value and a sour taste in the hybrid were more like the wild parent. The hybrid had a much higher value (4.5 ml) of titratable acidity than the control (1.2 ml).

Root-knot nematode resistance

The three groups (*C. hystrix*, *C. sativus*, and reciprocal interspecific hybrids) varied greatly in their response to *M. incognita*. While over 100 galls could be counted in each cucumber root system tested after 45 days, on average only three galls could be detected in each of the *C. hystrix* root systems. *C. hystrix* had a high level of resistance to *M. incognita* with mean gall index of 1.8. In contrast, cucumbers were confirmed as being highly susceptible possessing a mean gall index of 4.8–5.0. The interspecific F_1 hybrid was intermediate in resistance to the two parents, with a mean gall index 3.4. The transmission of resistance was observed in backcross progeny of the chromosome-doubled F_1 to cucumber. There was little variability in the mean gall index among of the individual , or the parental stocks.

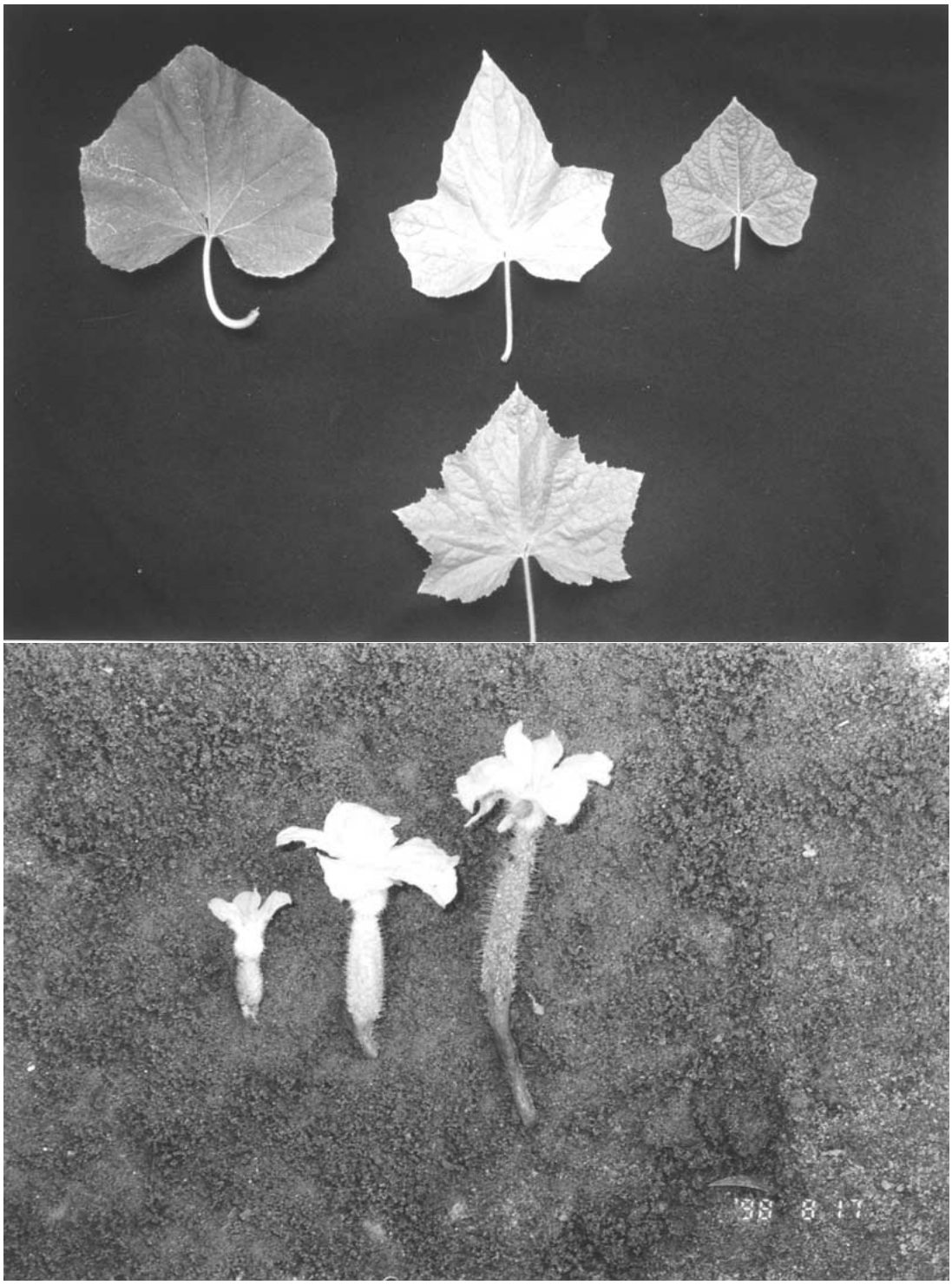
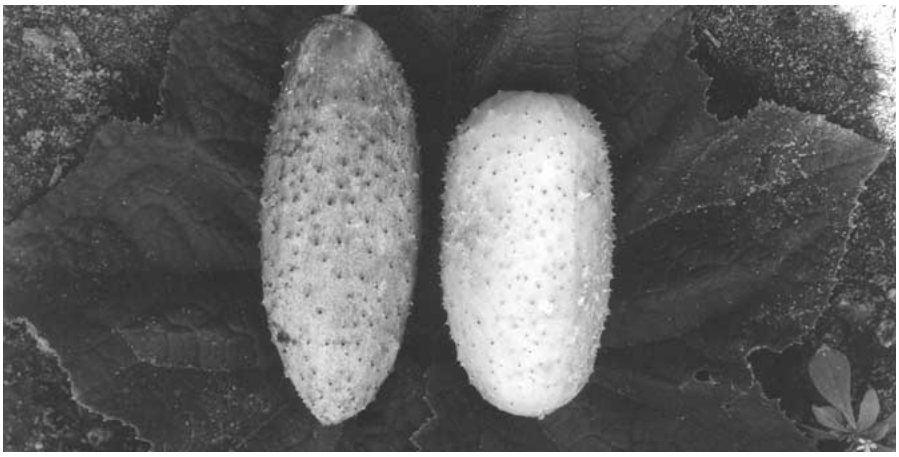


Figure 1. *a.* Leaves from the parental species and interspecific hybrid: *C. sativus* (upper left), interspecific hybrid with 2n = 19 (upper middle), *C. hystrix* (upper right), and the chromosome-doubled hybrid with 2n = 38 (lower middle).
b. Pistillate flowers in the cross: *C. hystrix* (left), interspecific hybrid (middle), *C. sativus* (right).
c. Fruits in the cross: *C. hystrix* (left), interspecific hybrid (middle), *C. sativus* (right).
d. The interspecific hybrid plants with different ploidy level: fruit diploid plant (left); in tetraploid plant (right).
e. Seeds in the cross: *C. sativus* (left), the amphidiploid (middle), *C. hystrix* (right).



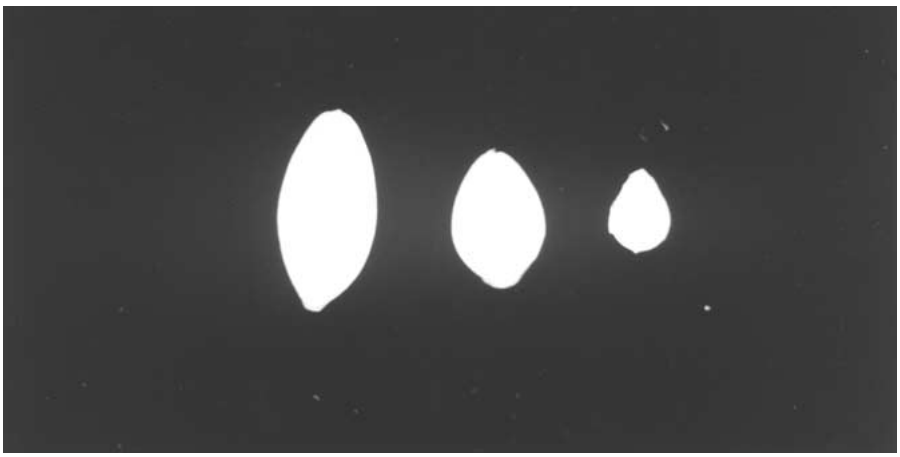


Figure 1. Continued.

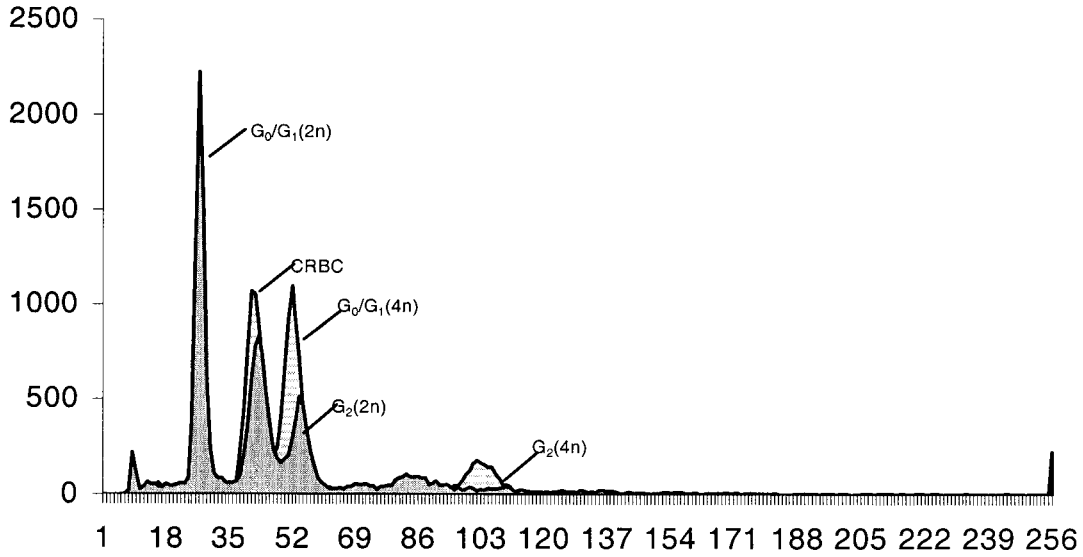


Figure 2. Histogram of number of nuclei per channel, a function of relative fluorescence intensity resulting from flow cytometry of nuclei stained with propidium iodide.

However, segregation for resistance was observed in the BC_1F_1 progeny.

Discussion

Fertility restoration in the chromosome-doubled F_1 hybrid plants marks the creation of a new synthetic *Cucumis* species which has a close phylogenetic relationship with its progenitor parents (cucumber and *C. hystrix*), but is distinct from both. The new species (amphidiploid) may be a vehicle for the transfer of desirable characteristics from *C. hystrix* to cucumber for the enhancement of pickling cucumber germplasm.

C. hystrix is a wild *Cucumis* species that develops small fruits with a fresh lemon flavor (it is eaten as wild fruit by the local minority). Since the progeny of the cross between cucumber and *C. hystrix* is edible, it could be considered a new crop. Two agriculturally novel products can be obtained directly from such an interspecific hybridization: 1) a new amphidiploid species, and; 2) a diploid seedless *Cucumis* vegetable. Compared with the cucumber parent, these new plants seem capable of producing more fruits (over 30 fruits/plant), and these fruits are more uniform (multiple fruit setting on a single node) than their *C. sativus* counterparts. Additionally, the fruits from the new species have higher nutrition value with regard to protein and mineral content. Also, they are resistant to pathogens such as powdery mildew, and tolerant to environmental stresses such as high temperature and low light intensity.

Table 2. Some nutrition factors

	Control*	Sample**
PH	5.9	4.5
Titratable acidity	1.2 ml	4.5 ml
Moisture	95.6%	96.4%
Ash	0.27%	0.35%
Protein	0.62%	0.78%
Carbohydrate	3.51%	2.47%

* Pickling cucumber bought from super market.
** *C. hystrix* × *C. sativus* ('Beijing Jietou') F_1 hybrid (2n = 19).

Natural resistance to the southern root-knot nematode, the major species causing severe yield loss of cucumber in many areas worldwide, is not available in cucumber. Resistance to *M. arenaria* and *M. javanica* was found in *C. sativus* L. var. *hardwickii* (Walters et al., 1993). In this study, the resistance to the southern root-knot nematode was identified in *C. hystrix*, and a potential for introgression of this resistance into cucumber was observed. Nevertheless, further experimentation is required to determine the inheritance of this character, and to define the strategies to facilitate the transmission and introgression of the resistance gene(s).

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